EXHIBIT A

UNITED STATES DISTRICT COURT

DISTRICT OF MASSACHUSETTS

IN RE: COLUMBIA UNIVERSITY PATENT LITIGATION	MDL NO. 04-01592
IMMUNEX CORPORATION, a Washington Corporation and AMGEN INC., a Delaware Corporation, Plaintiffs, vs. THE TRUSTEES OF COLUMBIA UNIVERSITY in the City of New York, a New York Corporation, Defendant.	Civil Action No. 04-10740-MLW C. D. Cal. No. CV 03-4349 MRP (CWx)
AND RELATED COUNTERCLAIM	

LETTERS ROGATORY (LETTER OF REQUEST) FOR ASSISTANCE WITH TAKING OF EVIDENCE AS TO DEPOSITIONS AND REQUESTS FOR DOCUMENTS OF THIRD PARTY WITNESS

TO: ONTARIO SUPERIOR COURT OF JUSTICE

The United States District Court requests international judicial assistance pursuant to the Ontario Evidence Act, R.S.O. 1990, c. E.23, s. 60 (1) and the Canada Evidence Act, R.S.C. 1985, c. C-5, s. 51; S.C. 1999, c. 18, s. 91. The request described herein is necessary in the interest of justice. The assistance requested is that the Ontario Superior Court of Justice require Dr. Louis Siminovitch (residence and place of business address below), to produce the documents set forth in Exhibit A attached hereto and to appear for a deposition, in accordance with the provisions of the Ontario Evidence Act, R.S.O. 1990, c. E.23, s. 60 (1) and the Canada Evidence Act, R.S.C. 1985, c. C-5, s. 51; S.C. 1999, c. 18, s. 91.

Residence:
130 Carlton Street
Toronto, ON M5A 4K3
Tel: (416) 975-5535

Place of Business:
Mount Sinai Hospital
600 University Ave.
Toronto, ON
M5G 1X5
Tel: (416) 586-8223

SYNOPSIS OF THE CASE

On December 10, 2003, plaintiffs Amgen Inc. and Immunex Corporation (collectively "Amgen") filed a declaratory judgment action in the Central District of California alleging that Amgen does not owe royalties and fees to the defendant, The Trustees of Columbia University ("Columbia"), under license agreements between Amgen and Columbia. The complaint further alleges that the only issued, non-expired, patent covered by the license agreements, U.S. Patent 6,455,275 ("the 275 patent"), issued September 24, 2002, is invalid and unenforceable. Columbia secured four patents from the U.S. Patent and Trademark Office regarding transformation technology, all based on the same patent application filed February 25, 1980, three of which expired in August of 2000. A fourth patent, the '275 patent, was issued in September 2002, claiming priority from the same patent application. Amgen alleges that the claims of the '275 patent are invalid for obviousness-type double patenting, among other grounds, and that the '275 patent is unenforceable under the doctrine of prosecution laches and inequitable conduct.

In April 2004, the United States Judicial Panel on Multidistrict Litigation consolidated Amgen's case with six other actions pending in Massachusetts, California, and New York, transferred the consolidated case to the U.S. District Court for the District of Massachusetts ("Massachusetts District Court"), and assigned the case to the Honorable Mark L. Wolf.

On June 23, 2004 the Massachusetts District Court carved out the issue of non-statutory double patenting as one that should be able to be quickly developed and decided in 2004 by establishing a schedule for the resolution of the non-statutory double-patenting issue in 2004.

On August 17, 2004, the Massachusetts District Court allowed Amgen's Motion for Discovery from Foreseeably Unavailable Witnesses, requesting the deposition of Dr. Louis Siminovitch. The Court allowed the deposition in order to preserve Dr. Siminovitch's testimony for trial.

Dr. Siminovitch's deposition is key to the case pending before the Massachusetts District Court because Dr. Siminovitch has personal knowledge of facts anticipated to be relevant to many issues in the case. For example, it is anticipated that his testimony will be relevant to the invalidity of the '275 patent under 35 U.S.C. §§ 102(a),(b),(f) & (g). Further, Dr. Siminovitch's testimony is relevant to prior art issues, double patenting and validity of the patent-in-suit because he made relevant prior art presentations, conducted studies, authored relevant prior art publications, and was in contact with one or more of the named inventors listed on the '275 patent and related patents.

The United States District Court for the District of Massachusetts has subject matter jurisdiction over this controversy pursuant to Title 35 of the United State Code, Section 1332 (a)(2).

CASE TYPE

This is a civil case.

NATURE OF THE ASSISTANCE REQUIRED

Assistance is requested with requiring Dr. Louis Siminovitch to produce documents and to appear for a deposition.

RECIPROCITY

The United States District Court for the District of Massachusetts is willing to provide similar assistance to judicial authorities of Ontario, Canada, in accordance with Title 28 of the United States Code, Section 1782.

REIMBURSEMENT FOR COSTS

Plaintiffs will pay the witness money and expenses as prescribed by the Ontario Superior Court of Justice.

Done at the United States District Court District of Massachusetts, 1 Courthouse Way, Boston, Massachusetts, 02210.

IT IS SO REQUESTED

Date: _____

_____
Honorable Mark L. Wolf
Judge for the United States District Court
For the District of Massachusetts

I, _____, am the clerk of the United States District Court of the District of Massachusetts and I certify that the Honorable Mark L. Wolf is a judge of the United States District Court for the District of Massachusetts and that he has laid his signature upon these Letters Rogatory.

Date: _____

_____
Clerk of the United States District Court
For the District of Massachusetts

[SEAL OF THE COURT]

I, Honorable Mark L. Wolf, am a judge of the United States District Court for the District of Massachusetts and I certify that _____ is the clerk of the United States District Court for the District of Massachusetts and that he has laid his signature and the seal of the Court upon these Letters Rogatory.

Date: _____

_____
Honorable Mark L. Wolf
Judge for the United States District Court
For the District of Massachusetts

EXHIBIT A TO SUBPOENA TO LOUIS SIMINOVITCH

INSTRUCTIONS

1. Pursuant to Federal Rule of Civil Procedure 45, plaintiff Amgen Inc. requests YOU to produce for inspection and copying the DOCUMENTS requested herein. Instead of producing such DOCUMENTS for inspection and copying at the location indicated in the subpoena, you may respond to this subpoena by mailing true and correct copies of all responsive DOCUMENTS to Vicki Norton at the offices of Wilson Sonsini Goodrich & Rosati, 3611 Valley Centre Drive, Suite 525, San Diego, California 92130, on or before the production date indicated on the subpoena.

2. All terms capitalized in these Requests and other terms not capitalized are defined in the definition section.

3. All terms capitalized in these Requests and other terms not capitalized are defined in the definition section or in the definitions set forth in Local Rule 26.5(c).

4. All DOCUMENTS, and all copies or drafts, shall be produced in their entirety in the order that they are kept in the usual course of business, and shall be produced in their original folders, binders, covers or containers, or photocopies thereof.

5. For each Request for which any DOCUMENT or object is withheld or not provided on the basis of any assertion of any testimonial or other privilege or doctrine, state the following: (1) the number of the Request; (2) the nature of the privilege or doctrine, *e.g.*, attorney-client privilege, work product doctrine or other privilege or doctrine; (3) a description of the DOCUMENT or object including (i) the author(s), addressee(s) and any indicated or blind copyee(s), (ii) the organizational position of the author(s), addressee(s) and any indicated or blind copyee(s), (iii) the DOCUMENT's date, number of pages and attachments or appendices, (iv) the subject matter(s) of the DOCUMENT, and (v) a description of the DOCUMENT or object sufficiently detailed to permit objective evaluation of the assertion of privilege, and (4) each and every fact upon which such assertion or privilege is based.

6. Each Request shall be answered fully unless in good faith objected to, in which event the reasons for YOUR objection shall be stated in detail. State the basis for YOUR objection and indicate what responsive DOCUMENTS or categories of DOCUMENTS YOU are producing in response to the Request. If an objection pertains to only a portion of a Request, or a word, phrase or clause contained within it, YOU are required to state YOUR objection to that portion only and respond to the remainder of the Request, using YOUR best efforts to do so. To the extent that YOU are objecting to any Request as vague and ambiguous, identify the terms, words, or language which YOU assert to be vague and ambiguous, and specify the meaning YOU are assigning to that term, word or language in YOUR response.

7. To the extent that YOU object to any Request on the ground that it calls for the confidential information of a third party, obtain permission from the third party to produce the confidential information and do not object on that basis.

DEFINITIONS

1. The definitions set forth in Local Rule 26.5(c) shall apply to these requests.

2. "YOU," "YOUR," or "YOURSELF" means and includes Louis Siminovitch and any other person or entity acting or purporting to act for or on behalf of you or who are subject to your direction and control, including but not limited to any attorneys, patent agents, foreign associates, agents, accountants, investigators, and consultants of any of the foregoing.

3. "CHO CELLS" means and includes any cells or cell lines derived from the ovaries of Chinese hamsters, whether wild-type or mutant, including but not limited to all cell lines from COLUMBIA, the University of Toronto, the University of Western Ontario, mutants of CHO CELLS, such as the A29 cell line, referenced in the '275 PATENT and CHO K1, DUKXB11, DXB11, DUKX or DG44 cell lines.

4. The connectives "and," "or," and "and/or" shall be construed either disjunctively or conjunctively, as necessary to bring within the scope of the discovery request all responses that might otherwise be construed to be outside of its scope.

5. The words "each," "every," "all," or "any" each include all of the words "each," "every," "all," and "any" as necessary to bring within the scope of the discovery request all responses that might otherwise be construed to be outside of its scope.

6. Whenever used herein, the singular shall include the plural and the plural shall include the singular.

7. Please note that these Requests include such DOCUMENTS and information within the custody, possession and control of YOU, YOUR subsidiary organizations, affiliates, and agents, including, but not limited to, YOUR respective counsel, accountant(s) and auditors.

REQUESTS FOR PRODUCTION

REQUEST FOR PRODUCTION NO. 1:

All DOCUMENTS and COMMUNICATIONS CONCERNING the transformation or cotransformation of any CHO CELLS or other eukaryotic cells between 1975 and the present.

REQUEST FOR PRODUCTION NO. 2:

All DOCUMENTS and COMMUNICATIONS CONCERNING "gene transfer" as that term is used in Lewis, W.H., *et al.*, "Parameters Governing the Transfer of the Genes for ThymidineKinase and Dihydrofolate Reductase into Mouse Cells Using Metaphase Chromosomes or DNA," *Somatic Cell Genet.*, 6(3):333-347 (1980).

**The referenced article is attached for your convenience.

REQUEST FOR PRODUCTION NO. 3:

All laboratory notebooks CONCERNING "gene transfer" as that term is used in Lewis, W.H., *et al.*, "Parameters Governing the Transfer of the Genes for ThymidineKinase and Dihydrofolate Reductase into Mouse Cells Using Metaphase Chromosomes or DNA," *Somatic Cell Genet.*, 6(3):333-347 (1980), or CONCERNING the transformation or cotransformation of CHO CELLS or other eukaryotic cells.

REQUEST FOR PRODUCTION NO. 4:

All DOCUMENTS filed with the Canadian Government or the National Archives of Canada CONCERNING any of YOUR work CONCERNING "gene transfer" as that term is used in Lewis, W.H., *et al.*, "Parameters Governing the Transfer of the Genes for ThymidineKinase and Dihydrofolate Reductase into Mouse Cells Using Metaphase Chromosomes or DNA," *Somatic Cell Genet.*, 6(3):333-347 (1980), or CONCERNING the transformation or cotransformation of CHO CELLS or other eukaryotic cells.

REQUEST FOR PRODUCTION NO. 5:

All DOCUMENTS and COMMUNICATIONS CONCERNING Parithychery R. Srinivasan's sabbatical(s) to the University of Toronto, including but not limited to all DOCUMENTS CONCERNING the work performed and any grants related thereto.

REQUEST FOR PRODUCTION NO. 6:

Copies of all of YOUR curriculum vitae (regardless of whether a recent version, a superceded version or a draft), including but not limited to all educational background, experience, training, list of publications, authorships, editorials, honors, and awards.

REQUEST FOR PRODUCTION NO. 7:

All DOCUMENTS CONCERNING any of YOUR publications, grants and/or presentations, including but not limited to drafts or notated copies thereof.

ment CAK-TK⁻ in the interferon system. These two hybrid lines are approximately 100X more sensitive than the parental CAK-TK⁻ line. One possibility for such complementation is that the interferon system of PCC4-aza 1 is activated in the hybrid cell. Such activation of an embryonal carcinoma cell gene has been reported for globin synthesis (16). The possibility of an activation of the interferon genes in PCC4-aza 1 cannot be verified in these intraspecific hybrids. We are presently investigating heterospecific hybrids to determine if such an activation can be detected.

ACKNOWLEDGMENTS

We thank Dr. F. H. Ruddle and R. M. Liskay for the cell lines used. This work has been supported by grant VC-244 from the American Cancer Society.

LITERATURE CITED

1. Kleinsmith, L. J., and Pierce, G. B. (1964). *Cancer Res.* **24**:1544–1551.
2. Pierce, G. B., Dixon, F. S., and Verney, E. L. (1960). *Lab. Invest.* **9**:583–602.
3. Papaioannou, V. E., McBurney, M. W., Gardner, R. L., and Evans, M. G. (1975). *Nature (Lond.)* **285**:70–73.
4. Illmensee, K., and Mintz, B. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73**:549–553.
5. Sherman, M. I., and Solter, D. (eds.) (1975). *Teratomas and Differentiation* (Academic Press, New York).
6. Swartzendruber, D. E., and Lehman, J. M. (1975). *J. Cell Physiol.* **85**:179–188.
7. Kelly, F., and Boccara, M. (1976). *Nature (Lond.)* **262**:408–411.
8. Burke, D. C., Graham, C. F., and Lehman, J. M. (1978). *Cell* **13**:243–248.
9. Jakob, H., Boon, T., Galiard, J., Nicolas, J.-F., and Jacob, F. (1973). *Ann. Microbiol. (Inst. Pasteur)* **124B**:269–282.
10. Dulbecco, R. and Vogt, M. (1954). *J. Exp. Med.* **99**:167–182.
11. Atkins, G. J., Johnston, M. D., Westmacott, L. M., and Burke, D. C. (1974). *J. Gen. Virol.* **25**:381–390.
12. Sherman, M. I. (1975). In *Teratomas and Differentiation* (eds.) Sherman, M. I. and Solter, D. (Academic Press, New York), pp. 189–205.
13. Miller, R. A., and Ruddle, F. H. (1976). *Cell* **9**:45–55.
14. Miller, R. A., and Ruddle, F. H. (1977). *Dev. Biol.* **56**:157–173.
15. McBurney, M. W. (1977). *Cell* **12**:653–662.
16. McBurney, M. W., Featherstone, M. A., and Kaplan, H. (1978). *Cell* **15**:1323–1330.
17. Litwak, G. and Croce, C. M. (1979). *J. Cell Physiol.* **101**:1–8.

Somatic Cell Genetics, Vol. 6, No. 3, 1980, pp. 333–347

Parameters Governing the Transfer of the Genes for Thymidine Kinase and Dihydrofolate Reductase into Mouse Cells Using Metaphase Chromosomes or DNA

William H. Lewis,[1] P.R. Srinivasan,[2] Nancy Stokoe,[1] and Louis Siminovitch[1]

[1]*Department of Medical Genetics, University of Toronto, Toronto, Canada M5S 1A8, and* [2]*Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York, 10027*

Received 31 July 1979—Final 8 November 1979

Abstract—*The conditions necessary to achieve high frequency transfer of the thymidine kinase and dihydrofolate reductase genes from hamster cells into mouse cells were investigated. Of the parameters examined, the length of adsorption time, input gene dosage, and treatment with dimethylsulfoxide (DMSO) were found to significantly alter the transfer frequency using either metaphase chromosomes or purified DNA as the transfer vehicle. With the mouse cell line as a recipient, the optimal adsorption period for DNA or chromosomes from Mtx^{RIII} cells was found to vary from 8 to 16 h in those experiments where the recipient cells were subsequently treated with DMSO. Without DMSO, similar frequencies could be obtained by extending the period of adsorption. Increasing the dosage of DNA or chromosomes resulted in an almost linear increase in the number of transformants. The optimal conditions for transfer did not significantly differ for the two genes studied. On the average, the optimal conditions yielded 1.5 × 10^3 transformants per 10^7 recipient cells with chromosomes; with DNA an average of only 60 transformants were observed. In general, DNA transformants grown in the absence of methotrexate were unstable; whereas, under the same conditions about 20% of the transformants from the chromosome experiments were stable.*

INTRODUCTION

Methods have been developed in recent years for the transfer of genetic material into cultured mammalian cells using either metaphase chromosomes or purified DNA. The genes which have been transferred by means of

0098-0366/80/0500-0333$03.00/0

chromosomes include those for thymidine kinase (tk) and hypoxanthine guanine phosphoribosyl transferase (hgprt), whereas success has been obtained, using DNA, for genes governing tk, hgprt, dihydrofolate reductase, and adenosine phosphoribosyl transferase (aprt) (1–7). One of us (L.S.) was involved as a co-author in a series of papers in which the transfer of a variety of other genes by means of metaphase chromosomes was described (8–12). We have not been able to repeat those experiments, and we have reason to believe that the technologies described in those papers do not result in successful gene transfer.

The reported frequencies of gene transfer utilizing either chromosomes or DNA have been relatively low (10^{-5} to 10^{-7}), and those experiments resulting in high frequency transfer have necessitated the use of the mouse L cell line as recipient (6, 7). In preliminary experiments we found that whereas high frequencies of gene transfer could be obtained with L cell recipients, such frequencies were several orders of magnitude lower with hamster recipient cell lines such as CHO or V79. The reasons for this are unknown. If gene transfer is to become an available and widely applied technique, then it will be necessary to improve the reproducibility and frequency of transfer both for L cells and for other cell types in which a variety of genetic markers are available for study. Since a number of variables are involved in gene transfer, and since no systematic study has been reported on the parameters which control the process, we have undertaken a comparative study of the transfer of two different genes using both metaphase chromosomes and DNA, and involving the examination of several different variables. We have used L cells as recipients for this work with the expectation that the results will be useful both in investigation of this system as well as for CHO and other cell systems.

MATERIALS AND METHODS

Cell Culture. Murine Ltk$^-$ clone D cells (13) provided by Dr. S. Silverstein, Columbia University were maintained in α-special medium (14) supplemented with 10% fetal bovine serum (FBS). Mtx^{RIII} is a Chinese hamster ovary (CHO) cell line isolated in two steps for resistance to methotrexate (15). In the first step cells were selected with a structurally altered dihydrofolate reductase (Mtx^{RI}) and the second step resulted in cells with increased levels of the altered enzyme. Mtx^{RIII} was routinely maintained in α-MEM (16) with 10% FBS.

Preparation of Chromosomes. Chromosomes were prepared by the procedure of Willecke and Ruddle (17) with some modifications. Mtx^{RIII} cells were grown in suspension to a density of 4×10^5 cells per milliliter and distributed in 50-ml aliquots into 20 flasks (150 cm^2). After 12 h at 37°C, 3.5

μg of colcemid (Sigma Chemical Co.) was added to each flask. After another 12 h of incubation at 37°C, the mitotic cells were detached by gently shaking five times. Approximately 90°C of the detached cells were in mitosis. The cells were centrifuged at 200*g* for 20 min, and resuspended in 50 ml of cold hypotonic (75 mM) KCl. After 15 min at 4°C, the swollen cells were centrifuged at 200*g* for 10 min. The pellets were resuspended at room temperature in 40 ml of 15 mM HEPES buffer, pH 7.0, containing 3 mM $CaCl_2$ and 0.5% Tween 80 and transferred to a glass Dounce homogenizer. The cells were disrupted by 6 to 10 strokes of the homogenizer, and the suspension was centrifuged at 100*g* for 8 min in plastic tubes to remove unbroken cells, nuclei, and other debris. At this stage a sample of the supernatant was placed in a haemocytometer and viewed under phase contrast optics. The number of chromosomes was counted and expressed as cell equivalents per milliliter.

As outlined here, the isolation procedure yielded from 40 to 100 $\times$ 10^6 cell equivalents of Mtx^{RIII} chromosomes. The amount of DNA in the samples was occasionally checked by the Dische diphenylamine reaction (18), and this value correlated well with the amount of DNA expected on the basis of chromosome cell equivalents. The supernatant solution was then transferred to four siliconized 15-ml glass tubes and centrifuged at 1300*g* for 20 min at 4°C. The pellet in each tube was resuspended by agitation in 10 ml of 15 mM HEPES buffer, pH 7.0 containing 3 mM $CaCl_2$ and again centrifuged at 1300*g* for 20 min at 4°C. The washed pellets were resuspended in 25 mM HEPES buffer, pH 7.1, containing 140 mM NaCl and 0.75 mM $Na_2HPO_4 \cdot 12\ H_2O$ at a concentration of 2 to 4 $\times$ 10^6 chromosome cell equivalents per milliliter. The buffered phosphate solution was prepared and the pH adjusted with 1 N NaOH immediately before use.

Chromosome Transfer Method. The recipient Ltk$^-$ cells logarithmically growing in stock flasks were trypsinized and plated at 2×10^6 cells per 75-cm^2 flask containing α-special medium with 10% FBS. After 24 h, the medium was aspirated from the recipient flasks and 10 ml of fresh medium at 37°C was added. To the chromosome preparation in HEPES–NaCl–Phosphate buffer at room temperature, $CaCl_2$ (2.5 M) was slowly added with mixing to a final concentration of 125 mM. One or two milliliter of this preparation was added immediately with a plastic pipette to the medium on the recipient cells. After an adsorption period in a 37°C-CO_2 incubator, the medium was aspirated and 40 ml of fresh medium added. When DMSO was employed, it was added to a final concentration of 10% directly into the chromosome-containing medium at the end of the adsorption period. After 30 min at 37°C, the medium containing DMSO was aspirated and replaced with 40 ml of fresh medium.

Isolation of DNA. Six liters of Mtx^{RIII} cells were grown in suspension to

a density of 5 to 7 × 10^5 cells/ml and centrifuged. The nuclei from these cells were isolated by swelling in hypotonic buffer followed by homogenization in a Dounce homogenizer. The crude nuclei were washed once with hypotonic buffer containing 0.5% Tween 80. The DNA from the nuclei was isolated essentially by the method described by Pellicer et al. (19), and dissolved in 1 mM TRIS-Cl, pH 7.9, containing 0.1 mM EDTA. The molecular weight of the purified DNA was at least 40 × 10^6 daltons as estimated after agarose gel electrophoresis. The concentration of the DNA was determined by the diphenylamine reaction.

DNA Transfer Method. The preparation of the calcium phosphate-DNA complex has been described (7,20). In order to obtain reproducible results, we have introduced several modifications which are described in detail here and elsewhere (21). 160 μg of purified Mtx^{RIII} DNA was gently dispersed at room temperature into a total volume of 3.6 ml of 1.0 mM TRIS-Cl, pH 7.9, containing 0.10 mM EDTA and stirred very slowly using a 1-cm Teflon coated magnetic bar in a 50-ml siliconized round bottom flask. $CaCl_2$ (2.5 M) was added to give a concentration of 250 mM. 4 ml of sterile HEPES buffer (50 mM), pH 7.1, containing 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 12\ H_2O$, prepared immediately before use, was introduced drop by drop along the side of the flask. After the addition, 1 or 2 ml of the precipitated solution was added immediately with a plastic pipette to recipient Ltk^- cells as described for chromosome transfer. After the adsorption period at 37°C, the medium was aspirated and replaced with 40 ml of fresh medium.

Expression and Selection. The flasks were incubated at 37°C for an additional 40 h after the adsorption period, at which time the cells were trypsinized and counted in a Coulter Counter. The number of cells per flask after expression was at least 1 × 10^7, all of which were plated for selection. The results of transfer experiments are expressed as the number of transformant colonies arising per 10^7 cells plated for selection.

Methotrexate resistant (Mtx^R) colonies were selected by plating 5 × 10^5 cells in 100-mm plates in α-special medium containing 10% dialyzed FBS and 2 × 10^{-7} M methotrexate (ICN Biochemicals). The methotrexate was freshly prepared in 0.01 N NaOH and the concentration after filtration was determined by measuring optical density at 257 nM and using an extinction coefficient of 23,000.

Cells possessing the enzyme thymidine kinase (tk^+) were selected by plating 1 × 10^6 cells in 100-mm plates in α-special medium containing 10% dialyzed FBS, 10 μg/ml hypoxanthine, 0.2 μg/ml of aminopterin, 5 μg/ml thymidine and 50 μg/ml of glycine (HAT medium). After incubation for 10 to 14 days at 37°C, the medium was removed and the colonies stained with

methylene blue. With these conditions, it was not necessary to renew the HAT medium every 3 or 4 days as reported (6). In addition to facilitating the selection, this procedure avoided the formation of satellite colonies which result from the disturbances of medium changes.

Untreated control Ltk^- cells plated in HAT medium or 2 × 10^{-7} M methotrexate resulted in no surviving colonies when 10^8 cells were plated. Control Ltk^- cells treated with homologous Ltk^- DNA or DNA from Mtx^s tk^- CHO cells likewise did not result in any colony formation when over 10^8 cells were plated under selective conditions. As expected, chromosomes from Mtx^s tk^+ CHO cells yielded an equivalent number of tk^+ transformants as chromosomes from the Mtx^{RIII} tk^+ donor cells used in this study. However, such chromosomes from Mtx^s hamster cells, when added to Ltk^- recipients, sometimes resulted in low frequency colony formation in 2 × 10^{-7} M methotrexate. For example, in one experiment Ltk^- cells treated with control Mtx^s hamster chromosomes, gave 20 Mtx^R colonies per 10^7 recipient cells. Under the same conditions however, Mtx^{RIII} chromosomes yielded over 3000 Mtx^R colonies per 10^7 cells (see Results). The nature of the low frequency Mtx^R colonies produced by the control chromosomes is not understood at present, but may be due to the mutagenic nature of added genetic material or to the transfer and amplification of the wild-type Mtx^s dihydrofolate reductase gene. Work is in progress to characterize this phenomenon.

Preparation of cell extracts and the assay of dihydrofolate reductase were carried out essentially as described by Flintoff et al. (15). Protein was measured by the method of Lowry et al. (22) using bovine serum albumin as the standard.

RESULTS

The process of gene transfer involves adsorption of the metaphase chromosomes or DNA to the recipient cells, the possible addition of adjuvants at appropriate times, and an expression period before selection. In the experiments to be described we have varied all of these parameters one at a time. As much as possible, for each experiment, the other variables were chosen to provide maximum efficiency, based on preliminary study.

Adsorption. Our first experiments were designed to investigate the optimal conditions for the exposure period to chromosomes or DNA. The donor CHO cells used for all of our experiments were auxotrophic for proline (pro^-), resistant to methotrexate (Mtx^{RIII}) and sensitive to BrdU (tk^+). In such a system, elimination of proline from the selection system controls against the possible transfer of intact donor cells. Since the recipient mouse

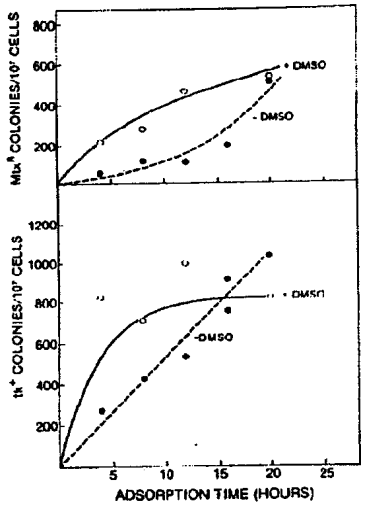

Fig. 1. The effect of length of exposure of Ltk$^-$ cells to chromosomes from Mtx^{RIII} cells on the frequency of transfer of the tk$^+$ and Mtx^R markers.

cells were methotrexate sensitive and tk$^-$, the transfer of both of these markers could be studied in parallel experiments using the same chromosome or DNA preparation.

The results using chromosomes and adsorption periods of 4 to 20 h are shown in Fig. 1. Based on experiments to be described later, DMSO was used at a concentration of 10% for 30 min and was added at the end of the adsorption period as described under Materials and Methods. The time allowed for expression of the genes before selection was 40 h from the end of the adsorption period. Representative results are shown in Figs. 1 and 2 for chromosome and DNA transfer, respectively. In most cases, the numbers of tk$^+$ and Mtx^R colonies rose with the increasing times of adsorption. With chromosomes, the optimal exposure time appears to depend upon whether the cells are treated with DMSO. In the absence of DMSO, the frequency of transformants in this experiment seemed to be increasing even at 20 h, although in other experiments the numbers had levelled off by this time. With DMSO, the levels reached a plateau at about 8 h for the tk marker and a little later for Mtx^R. As can be seen in Fig. 1, the frequencies with DMSO were nearly always greater than without the adjuvant, particularly at 8 to 12 h, the adsorption times which we have used routinely in most other experiments.

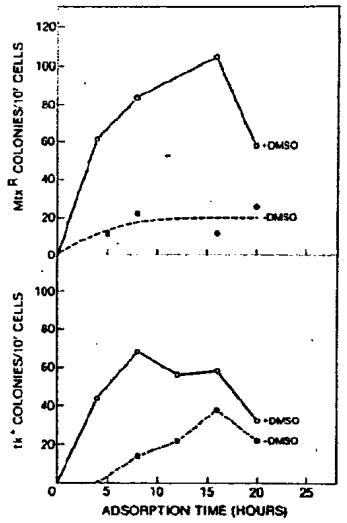

Fig. 2. The effect of length of exposure of Ltk$^-$ cells to DNA from Mtx^{RIII} cells on the frequency of transfer of the tk$^+$ and Mtx^R markers.

The data using DNA for transfer (Fig. 2) was similar in general. Levelling off was again observed, and the frequencies were always higher when DMSO was employed as adjuvant. Comparison of Fig. 1 and 2 illustrates a finding that we have observed consistently in our studies: the frequencies were always higher (10- to 100-fold) for chromosome versus DNA transfer, with or without DMSO.

Based on the data shown in Figs. 1 and 2, we have used an adsorption time of 8 to 12 h in all further work described here. Although longer adsorption times, especially in the absence of DMSO treatment, sometimes results in higher frequencies of transfer, the exposure of the recipient L cells to the calcium phosphate precipitate for long periods of time results in considerable cell killing. At 8 to 12 h of adsorption the amount of cell death is minimal, and thus larger numbers of transformants are obtained.

Gene Dosage. In developing a system for gene transfer, it is of course important to determine the relationship between dosage and the frequency of transfer and, at the same time, the amount of added material which will yield the highest number of transformants. The results of experiments to examine these questions are shown in Figs. 3 and 4 for chromosome and DNA transfer, respectively. In both cases the adsorption period used was 8 h and

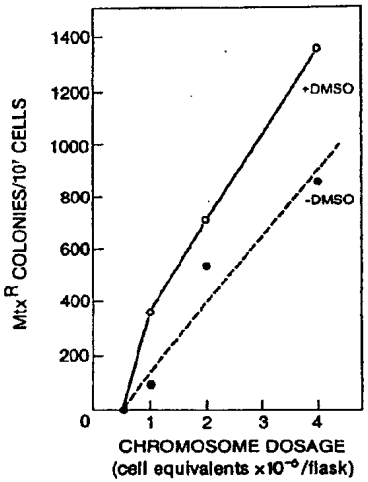

Fig. 3. The effect of varying chromosome dosage on the transfer of the Mtx^R marker.

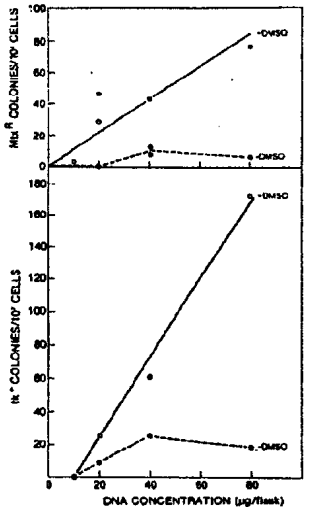

Fig. 4. The effect of varying DNA concentration on the frequency of transfer of the tk^+ and Mtx^R markers.

the expression time was 40 h. As can be seen, with DMSO treatment, the number of transformants observed increased markedly in proportion to the concentration of chromosomes or DNA. A similar increase was observed without DMSO when chromosomes were employed. With DNA, however, there was some increase in numbers of tk^+ or Mtx^R colonies observed without DMSO, but the curves levelled off at about 40 µg/ml. From the results of these experiments, the importance of using DMSO, especially for DNA transfer, is evident. As in the data shown in Figs. 1 and 2, it is again clear if one compares the numbers of Mtx^R colonies, that chromosome transfer yields a larger number of transformants than DNA. With 4×10^6 cell equivalents of CHO chromosomes per flask seeded with 2×10^6 LtK Mtx^S cells, the number of Mtx^R colonies scored reached 1400 per 10^7 recipient cells, a frequency of approximately 10^{-4}.

Although the frequencies of transformants, especially in the presence of DMSO, increase with the amount of genetic material added, there are practical limitations on the amount of material one can use. These limitations involve difficulties in preparing large amounts of chromosomes in a reasonable length of time and, more important, problems of viscosity and sticking when handling high concentrations of DNA or chromosomes in small volumes. We have found that concentrations of 4×10^6 cell equivalents of chromosomes and 40 µg of DNA per recipient flask yield high frequencies of transfer with a minimum of practical difficulties and these concentrations were employed throughout the experiments described here.

DMSO Treatment. The utility of DMSO treatment during the transfer of chromosomes had been indicated earlier by the experiments of Miller and Ruddle (2). Our experiments described in Figs. 1–4 confirm their conclusions and extend it to DNA. Because of the utility of DMSO addition, we examined whether important variations in the frequency of transformants would be observed if the time of DMSO contact was varied. This experiment was done using only DNA because DMSO plays a much more important role in this case (Figs. 1–4). DMSO up to a final concentration of 10% was added at 8 h after the addition of the DNA, allowed to remain in contact with the cells for various lengths of time, then removed, and 40 h after the end of the adsorption period, the cells were plated for selection of tk^+ transformants. As seen in Fig. 5, there is a linear relationship between the time of DMSO contact and the numbers of transformants. At first glance, it would appear that there is considerable advantage in leaving the DMSO in contact with the recipient cells over long periods. However, DMSO is toxic, and beyond 30 min there is appreciable cell killing. We have therefore adopted 30 min as our treatment time with 10% DMSO. We have not examined the effects of different concentrations of DMSO in detail, but again, the frequency of

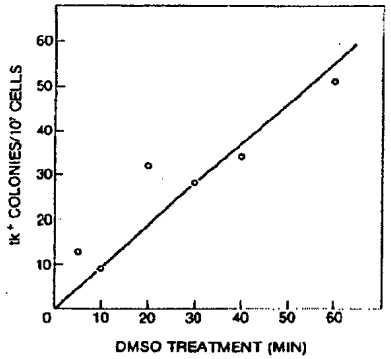

Fig. 5. Time course for treatment with DMSO. Ltk⁻ cells were exposed to 40 μg of DNA for 8 h and 10% DMSO was then added for the time periods indicated.

transformants seems to increase with increasing DMSO concentration (data not shown). Beyond 10% DMSO, cell killing becomes appreciable and there is therefore no significant advantage in using higher concentrations of this adjuvant.

Expression Time. Very little study has been done on the expression time

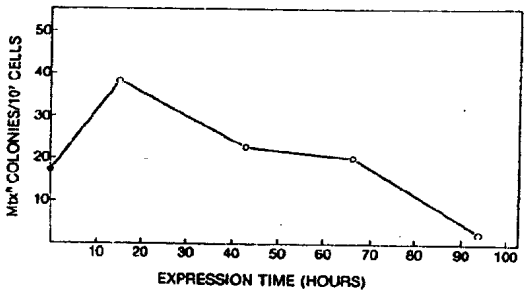

Fig. 6. The effect of variation of expression time on the frequency of transfer of Mtx^R marker. Five separate flasks seeded with 2×10^6 Ltk^- cells were exposed to 40 μg of Mtx^{RIII} DNA under standard transfer conditions. After DMSO treatment, the medium was removed and fresh medium introduced. Immediately, and at 15, 43, 66, and 94 h after DMSO treatment, the cells were trypsinized and plated in Mtx^R selective medium. For the later time points (66 and 94 h) the cells were trypsinized and diluted into fresh medium to maintain logarithmic growth during the extended expression time.

required for genetic material transferred from cell to cell. We have therefore examined the importance of this parameter using DNA as the vehicle and measuring transfer of the Mtx^R marker. Five separate flasks seeded with 2×10^6 Ltk^- cells were exposed to 40 μg of Mtx^{RIII} DNA for 8 h, followed by treatment with 10% DMSO for 30 min. The DMSO was then removed and fresh medium was introduced. Immediately, and at 15, 43, 66, and 94 after DMSO treatment, the cells were trypsinized and plated in Mtx^R selective medium. As shown in Fig. 6, there is no major effect of expression time; the difference in transfer frequency between 0 and 66 h being at the most two-fold. The large decrease in the number of transformants at 94 h corresponds to the resumption of logarithmic cell division and may reflect the instability of the transferred genetic material within the dividing cells. Because of the relatively minor effects of expression time up to 60 h, we have continued to use 40 h as a routine expression period.

Stability and Characterization of the Transformants. Previous studies in other laboratories have demonstrated that transformants are unstable when grown under nonselective conditions, but that stable lines can be developed by culture in selective medium (2,7). This type of investigation, however, has never been done in a comparative way by examining the fate of the same marker using chromosomes and DNA in the same system. We have carried out such experiments using the Mtx^{RIII} marker.

In Fig. 7 we show representative plates containing Mtx^R transformants obtained from a chromosome and DNA transfer experiment. It is immediately obvious that the sizes of the colonies are much larger when chromosomes are employed than when DNA is used. As will be seen shortly, this is almost surely a reflection of the greater stability of the transferred material derived from chromosomes.

We picked 5 colonies at random from plates in which the Mtx^R marker had been transferred by chromosomes and an additional 12 colonies from plates treated with DNA, and compared their stability in nonselective medium. When grown to 10^7 cells in the presence of 1×10^{-7} methotrexate, and then for three to four generations in the absence of drug, none of the 12 Mtx^R colonies derived from DNA transfer were subsequently able to form colonies when at least 10^4 cells were plated in 2×10^{-7} M methotrexate; this indicates their great instability under nonselective conditions. In contrast, Mtx^R colonies obtained after chromosome transfer were much more stable. As shown in Table 1, two of the five clones isolated retained significant resistance to 2×10^{-7} M methotrexate even after growth for 48 days in the absence of the drug. The remaining three transformant clones showed varying degrees of loss of the Mtx^R phenotype (Table 1).

In order to characterize the dihydrofolate reductase activity present in

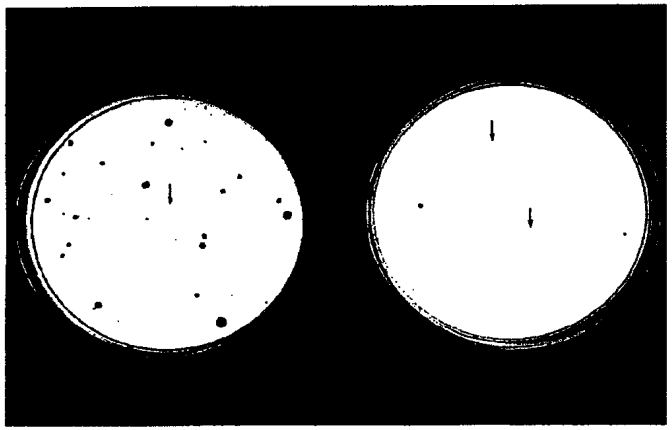

Fig. 7. Left, Mtx^R colonies from a chromosome transfer experiment. Right, Mtx^R colonies from a DNA transfer experiment. On each 100-mm plate, 5×10^5 Ltk^- cells that had been treated with 4×10^6 cell equivalents of Mtx^{RIII} chromosomes (left) or 40 μg of Mtx^{RIII} DNA (right) were plated in medium containing 2×10^{-7} M methotrexate. After 11 days incubation at 37°C, the medium was removed and the colonies stained with methylene blue. The arrows indicate the presence of small colonies.

the transformant clones, cell extracts were prepared from several transformants as well as from donor Mtx^{RIII} and recipient Ltk^- cells. As shown in Table 2, under our culture and assay conditions, the Mtx^{RIII} donor cells had almost 25-fold more dihydrofolate reductase activity when compared to the recipient Ltk^- cells. As expected, the enzyme activity from the Mtx^{RIII} cells was also 15-fold more resistant to inhibition by methotrexate. Extract from a

Table 1. Stability of Mtx^R Phenotype

Transformant clone	Plating efficiency in methotrexate[a]		
	6 days[b]	22 days	48 days
75-L-1	0.33	0.32	0.28
75-L-2	0.58	0.32	0.20
75-L-3	0.73	0.34	0.06
75-L-4	0.11	0.05	0.003
75-L-6	0.83	0.36	0.01

[a] Figures are given as the plating efficiency in the presence of 2×10^{-7} M methotrexate divided by the plating efficiency in the absence of the drug.
[b] Methotrexate-resistant colonies were picked from a chromosome transfer experiment and grown to approximately 2×10^7 cells in 1×10^{-7} M methotrexate, at which time they were transferred to medium lacking methotrexate for the specified number of days.

Table 2. Dihydrofolate Reductase Activity of Transformant Clones

Cell type	Relative I_{50}[a]	Relative specific activity[b]	RPE in Mtx[c]
Ltk^-	1.0	1.0	$<1 \times 10^{-4}$
Mtx^{RIII}	15.0	24.8	1.0
75-L-2	12.5	2.6	0.2
75-L-6 (t_4)[d]	22.0	13.3	0.72
75-L-6 (t_{48})[d]	0.9	1.2	0.01

[a] Concentration of methotrexate required to inhibit dihydrofolate reductase activity by 50% relative to an I_{50} of 4×10^{-9} M for Ltk^-.
[b] Decrease in absorbance at 340 nm/min mg^{-1} of extract protein, relative to a specific activity of 0.016 OD/min mg^{-1} for Ltk^-.
[c] Relative plating efficiency in α-special medium containing 10% dialyzed FBS and 2×10^{-7} M methotrexate.
[d] Figure in brackets refers to the number of days cells were cultivated in the absence of methotrexate (see Table 1).

stable transformant (75-L-2) contained approximately 3-fold more dihydrofolate reductase activity than the untransformed recipient cells and the methotrexate resistance of the enzyme was comparable to that observed in the donor Mtx^{RIII} cells (Table 2). After only short periods of growth in nonselective medium, an unstable transformant (75-L-6) also possessed an elevated level of the enzyme with similar resistant properties as the donor dihydrofolate reductase. However, Table 2 shows that prolonged growth in the absence of methotrexate reduced both the level and resistance of the dihydrofolate reductase to that found before transfer. This property was reflected in a reduced ability of these cells to form colonies in methotrexate containing medium.

DISCUSSION

The present study was undertaken to develop reproducible methods which would yield consistently high frequency gene transfer with chromosomes or DNA, and to compare the transfer frequencies in the two systems. We have found that three major parameters can affect transformation frequency. These are adsorption time, gene dosage, and treatment with DMSO postadsorption.

The enhancement of chromosome gene transfer by DMSO confirms the earlier results by Miller and Ruddle (2). However, we have found that the effect of DMSO is much more striking when DNA is the gene transfer vehicle. Although DMSO is known to act on a variety of biological processes, its biochemical mechanism(s) of action on gene transfer is not known. DMSO stimulates erythroid differentiation (23), seems to affect the nature of histone subfractions (24), and has been shown to elicit an endodeoxyribonuclease

activity responsible for single strand cleavage of cellular DNA (25,26). DMSO also increases plaque formation in BHK cells infected with HSV-1 DNA in the presence of calcium phosphate (27). While DMSO may influence the properties of the cell membrane as indicated by the enhanced cell fusion with polyethyleneglycol (28), and decreased membrane fluidity (29), it is tempting to speculate that DMSO promotes DNA repair mechanisms and thus facilitates integration of foreign DNA.

As indicated earlier, we have consistently found a much higher frequency for transfer of the tk and Mtx^R markers when we have used chromosomes. Under similar conditions of 8 h adsorption and 10% DMSO treatment for 30 min in five separate experiments, the frequency of chromosome transfer of the Mtx^R marker was 1.5×10^{-4}, whereas that for DNA transfer was 6.4×10^{-6}. The lower frequency observed with DNA may be due to a smaller size of the genetic fragment transferred. The Mtx^{RIII} cell line has been shown recently to contain an approximately 10-fold amplification in the number of genes for dihydrofolate reductase (R. Axel, personal communication). Work is in progress to determine how many copies of the resistant Mtx^{RIII} dihydrofolate reductase gene need to be transferred in order for the transformant cells to grow under the selective conditions. Large size colonies and stability of the chromosome transformants could also imply greater efficiency of integration whereas smaller colonies and instability could be a consequence of unstable integration or abortive transformation. If this supposition is correct, then increases in frequency either with DNA or chromosomes may require the development of methodologies which promote the stable integration of genetic material.

Comparison of our transfer frequencies with those reported from other laboratories is difficult because experimental procedures and the methods used to calculate transfer frequency differ. The highest frequency of chromosome-mediated transfer seems to be about 10^{-5}, reported by Miller and Ruddle for the hgprt marker. Wigler et al., (7) have reported almost as high frequencies for DNA transfer of the tk and aprt genes. With the changes introduced in this paper, we have markedly enhanced transfer frequency by both chromosomes and DNA; under our conditions, gene transfer can routinely be achieved at 5- to 20-fold higher frequencies than those reported in their studies.

Work in other labs as well as our own has indicated that successful gene transfer is much more difficult when Chinese hamster ovary cells are used as recipients instead of Ltk^- cells, and in fact no positive report has appeared describing such transfer. Recently, we have been able to transfer Mtx^R, tk^+, as well as the genes for ribonucleotide reductase (30,31) and leucyl tRNA synthetase (32) into Chinese hamster cells by chromosome transfer, albeit with a much lower frequency than that obtained with L cells. Because of the large spectrum of markers available in the CHO cell line, it will be important to attempt further enhancement of these transfer efficiencies using the methods outlined in this paper.

ACKNOWLEDGMENTS

We wish to thank Dr. David Hoar for kindly providing laboratory facilities and encouraging discussion during the course of this study, W.H.L. and P.R.S. also take this opportunity to thank Carol Miller and Dr. Frank Ruddle for the helpful discussions during their visit to Dr. Ruddle's laboratory.

The research was supported by grants to L.S. from the Medical Research Council and National Cancer Institute of Canada and the National Institutes of Health, U.S.A. W.H.L. was the recipient of a Medical Research Council of Canada Fellowship. P.R.S. was the recipient of a Senior International Fellowship from the Fogarty International Center, National Institutes of Health, U.S.A.

LITERATURE CITED

1. McBride, O. W., and Ozer, H. L. (1973). *Proc. Natl. Acad. Sci. U.S.A.* 70:1258–1262.
2. Miller, C. L., and Ruddle, F. H. (1978). *Proc. Natl. Acad. Sci. U.S.A.* 75:3346–3350.
3. Willecke, K., Lange, R., Kruger, A., and Reber, T. (1976). *Proc. Natl. Acad. Sci. U.S.A.* 73:1274–1278.
4. Wullems, G. J., Van Der Horst, J., and Bootsma, D. (1977). *Somat. Cell Genet.* 3:281–293.
5. McBride, O. W., Burch, J. W., and Ruddle, F. H. (1978). *Proc. Natl. Acad. Sci.* 75:914–918.
6. Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978). *Cell* 14:725–731.
7. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979). *Proc. Natl. Acad. Sci.* 76:1373–1376.
8. Spandidos, D., and Siminovitch, L. (1977). *Proc. Natl. Acad. Sci. U.S.A.* 74:3480–3484.
9. Spandidos, D., and Siminovitch, L. (1977). *Cell* 12:235–242.
10. Spandidos, D., and Siminovitch, L. (1977). *Cell* 12:675–682.
11. Spandidos, D., and Siminovitch, L. (1977). *Brookhaven Symposium Biology* 29:127–134.
12. Spandidos, D., and Siminovitch, L. (1978). *Nature* 271:259–261.
13. Kit, S., Dubbs, D., Piekarski, L., and Hsu, T. (1963). *Exp. Cell Res.* 31:291–312.
14. McBurney, M. W., and Whitmore, G. F. (1974). *Cell* 2:173–182.
15. Flintoff, W. F., Davidson, S. V., and Siminovitch, L. (1976). *Somat. Cell Genet.* 2:245–261.
16. Stanners, C. P., Elicieri, G. L., and Green, H. (1971). *Nature (London)* 230:52–54.
17. Willecke, K., and Ruddle, F. H. (1975). *Proc. Natl. Acad. Sci. U.S.A.* 72:1792–1796.
18. Burton, K. (1956). *Biochem. J.* 62:315–323.
19. Pellicer, A., Wigler, M., Axel, R., and Silverstein, S. (1978). *Cell* 14:133–141.
20. Graham, F. L., and van der Eb, A. J. (1973). *Virology* 52:456–567.
21. Srinivasan, P. R., and Lewis, W. H. (1980). In *Introduction of Macromolecules into Viable Mammalian Cells* (ed.) Baserga, R., Croce, C., and Rovera, G. (Alan R. Liss, New York). Wistar Symposium Series 1:27–45.

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* 193:265–275.
23. Friend, C., Scher, W., Holland, J. G., and Sato, T. (1971). *Proc. Natl. Acad. Sci. U.S.A.* 68:378–382.
24. Terada, M., Fried, J., Nudel, Y., Rifkind, R. A., and Marks, P. (1977). *Proc. Natl. Acad. Sci. U.S.A.* 74:248–252.
25. Scher, W., and Friend, C. (1978). *Cancer Res.* 38:841–849.
26. Terada, M., Nudel, U., Fibach, E., Rifkind, R. A., and Marks, P. (1978). *Cancer Res.* 38:835–840.
27. Stow, N. D., and Wilkie, N. M. (1976). *J. Gen. Virol.* 33:447–458.
28. Norwood, T. H., Ziegler, C. J., and Martin, C. M. (1976). *Somat. Cell Genet.* 2:263–270.
29. Lyman, G. H., Preisler, H. D., and Papahadjopoulos, D. (1976). *Nature* 262:360–363.
30. Lewis, W. H., and Wright, J. A. (1978). *J. Cell. Physiol.* 97:87–98.
31. Lewis, W. H., and Wright, J. A. (1979). *Somat. Cell Genet.* 5:83–96.
32. Thompson, L., Harkins, J., and Stanners, C. (1973). *Proc. Natl. Acad. Sci. U.S.A.* 70:3094–3098.

Somatic Cell Genetics, Vol. 6, No. 3, 1980, pp. 349–359

Rapid, Quantitative Analysis of Cell Cycle Stages of Cold-Sensitive Derivatives of the Chinese Hamster Cell Line CHO-K1

B. M. Ohlsson-Wilhelm,[1,3] J. F. Leary,[2] M. Pacilio,[1] and T. Martin[1]

Departments of [1]Microbiology and [2]Pathology, University of Rochester, Rochester, New York 14642

Received 2 October 1979—Final 30 January 1980

Abstract—*Cell cycle parameters of two cold-sensitive Chinese hamster cell lines have been determined by flow cytometric analysis of cell populations stained with the DNA specific fluorochrome mithramycin. The most striking finding is a depletion of S phase cells, detectable as early as 12 h after a shift to the nonpermissive temperature of 30.0°C and complete by 24 h following the shift. There is a substantial increase in the proportion of cells in the G1 phase of the cell cycle compared to wild type cells under identical conditions but, surprisingly, the proportion of cells having a G2/M DNA content is quite similar in the two populations. The proportion of tetraploid cells present in these populations is not sufficient to account for this observation. Reversibility of the cold-induced block was tested by returning cells held for three days at the nonpermissive temperature to the permissive temperature. Cells having a G1 content of DNA do reenter the S phase, beginning approximately 8 h after a return to the permissive temperature.*

INTRODUCTION

Temperature-sensitive mutants have proven extremely useful for the determination of structure–function relationships (1) and for timing the occurrence of specific functions in developmental sequences, both at the level of cells (2) and in whole organisms (3). This class of conditionally lethal mutants divides naturally into two parts: the heat sensitives and the cold sensitives. A wide variety of heat-sensitive mutants has been derived from

[3]Address all correspondence to: Dr. Betsy M. Ohlsson-Wilhelm, University of Rochester, Medical Center Box 672, Rochester, New York 14642.